The gene for trypsin inhibitor CMe is regulated in *trans* by the *lys 3a* locus in the endosperm of barley (*Hordeum vulgare* L.)

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Summary. A cDNA encoding trypsin inhibitor CMe from barley endosperm has been cloned and characterized. The longest open reading frame of the cloned cDNA codes for a typical signal peptide of 24 residues followed by a sequence which is identical to the known amino acid sequence of the inhibitor, except for an Ile/Leu substitution at position 59. Southern blot analysis of wheat-barley addition lines has shown that chromosome 3H of barley carries the gene for CMe. This protein is present at less than 2%–3% of the wild-type amount in the mature endosperm of the mutant Risø 1508 with respect to Bomi barley, from which it has been derived, and the corresponding steady state levels of the CMe mRNA are about 1%. One or two copies of the *CMe* gene (synonym *Itc1*) per haploid genome have been estimated both in the wild type and in the mutant, and DNA restriction patterns are identical in both stocks, so neither a change in copy number nor a major rearrangement of the structural gene account for the markedly decreased expression. The mutation at the *lys 3a* locus in Risø 1508 has been previously mapped in chromosome 7 (synonym 5H). A single dose of the wild-type allele at this locus (*Lys 3a*) restores the expression of gene *CMe* (allele *CMe-1*) in chromosome 3H to normal levels.

Key words: Trypsin inhibitor – Gene regulation – *lys 3a* locus – Barley

Introduction

Mutations affecting the overall lysine content of cereal endosperm have received considerable attention because lysine is the limiting essential amino acid in cereal grains when used as food for monogastric animals (Mertz et al. 1964; Nelson et al. 1965; Munck et al. 1970; Singh and Axtell 1973; Ingversen et al. 1973; Doll 1973, 1983, 1984). However, these so-called high-lysine mutations do not specifically affect lysine biosynthesis and their effect on lysine content is the indirect result of profound alterations in the pattern of protein accumulation throughout development and, at least in some cases, they also affect the metabolism of carbohydrates and lipids, as well as seed morphology. A common feature of many of these mutants is a decrease in the level of storage prolamins and it has been suggested that they should be considered as low-prolamin rather than high-lysine mutants (Doll 1984). In barley, three such mutations have been most extensively studied: (1) a spontaneous mutation in the *lys* locus of Hiproly barley, which was identified in a screening of the world barley collection (Munck et al. 1970); (2) a mutation affecting the *lys 3a* locus in mutant Risø 1508, which was induced experimentally from cv. Bomi (Doll et al. 1974); and (3) the *Hor-2ca* mutation in Risø 56, obtained by γ-ray treatment of Carlsberg II barley (Doll 1980). While a major portion of the B hordein structural genes at the *Hor-2* locus has been deleted in Risø 56 (Kreis et al. 1983), in the other two mutations, both of which map in separate loci of chromosome 7 (synonym 5H; Karlsson 1976, 1977), *trans*-acting regulatory genes seem to be affected (Doll 1984). The *lys 3a* mutation drastically reduces the net accumulation of all hordein polypeptides except D-hordein (Ingversen et al. 1973; Hopp et al. 1983; Thompson and Bartels 1983; Kreis et al. 1984). The reduction in storage protein accumulation seems to bring about an increase in the synthesis of other polypeptides and in the level of free amino acids (Brandt 1976; Shewry et al. 1980). Hiproly and its high-lysine derivatives have also been reported to have a reduced prolamin production, although to a lesser extent than in Risø 1508, and a significant increase in other proteins, such as protein Z, β-amylase, and two chymotrypsin inhibitors, CI-1 and CI-2 (Hejgaard and Boisen 1980; Boisen et al. 1981; Hejgaard 1982). In contrast, the genes for protein Z and β-amylase are almost completely switched off by the *lys 3a* mutation (Balasaraswathi et al. 1984; Kreis et al. 1987; Sørensen et al. 1989). We report here the molecular cloning of the cDNA corresponding to trypsin inhibitor CMe and present evidence that gene *CMe* (synonym *Itc1*; see Søgaard and von Wettstein-Knowles 1987) is regulated in *trans* by the *lys 3a* locus.

Materials and methods

Biological material. The cultivated barley *Hordeum vulgare* cv. Bomi and its mutant Risø 1508 were the gift of H. Doll (Risø Laboratory, Denmark). Disomic addition lines of Betzes barley in Chinese Spring wheat were donated by K. Shepherd (Adelaide, Australia). Barley cv. Hatif de Grignon was from La Cruz del Campo SA (Sevilla, Spain), where the crosses with Risø 1508 were carried out. A cDNA

library from the endosperm of *Hordeum vulgare* cv. Abissynian 2231 in the vector λNM1149 was the kind gift of W. Rohde (Max Planck Institute, Köln, FRG). B-hordein clone pB11 was provided by Dr. J. Forde (Rothamsted, UK).

Screening of the cDNA library. The library was screened with a 17 nucleotide degenerate synthetic probe as described by Woods et al. (1982). Prehybridization was in 6 × SSC, 1 × Denhardt's solution, 0.5% SDS, 0.05% sodium pyrophosphate, 100 μg/ml herring sperm DNA at 37° C for 2 h Hybridization was done overnight at the same temperature. The oligonucleotide was labeled with T4 polynucleotide kinase. The filters were washed at 37° C for 1 h with 3 × SSC, 0.05% sodium pyrophosphate, followed by a stringent wash at 47° C for 10 min. Large-scale preparation of purified recombinant phages was carried out according to Yamamoto et al. (1970).

DNA sequencing. DNA sequencing was carried out both by the dideoxy chain-termination method of Sanger et al. (1977) and by the chemical modification method of Maxam and Gilbert (1980). Appropriate fragments were subcloned in the M13mp18/mp19 or in the pUC18/19 vector systems as required.

Dot-blot analysis and filter hybridization. Isolation of DNA from dark-grown seedlings was according to Murray and Thompson (1980). Restriction digestions, agarose gel electrophoresis and Southern blotting to nylon membranes (Hybond N, Amersham) were performed by standard techniques (Maniatis et al. 1982) or according to the manufacturer's instructions. Hybridization with multiprimer-labeled probes (Feinberg and Vogelstein 1983) was in 5 × SSPE, 2 × Denhardt's solution, 0.2% SDS, 10 μg/ml herring sperm DNA at different temperatures.

Total RNA from developing endosperms and from coleoptiles was obtained by the hot-phenol procedure. Quantitation of CMe mRNA was by standard dot-blot analysis or by blotting the appropriate clones and hybridization with ^{32}P-labeled total RNA as a probe (Domoney and Casey 1987).

Protein fractionation and analysis. Individual endosperms (ca. 30 mg) were delipidated and extracted with 0.5 M NaCl as previously described (Salcedo et al. 1984). The extracts were fractionated by high-performance liquid chromatography (HPLC) or by two-dimensional electrophoresis.

Fractionation by HPLC was on a Vydac TP-RP-C4 column at 1 ml/min with the following composite acetonitrile/H_2O linear gradient: starting buffer, 10% acetonitrile (0.1% trifluoroacetic acid); up to 20% in 45 min; 20%–35% in 140 min; 35–50% in 100 min; 50%–85% in 12 min.

Fractionation by two-dimensional electrophoresis was essentially as follows: the first separation was by electrofocusing (IEF; ampholines pH 4–9, 5% polyacrylamide; 2 mm × 14 mm columns; 7 h at 470 V; samples inserted at acid end) and the second separation was by starch gel electrophoresis (SGE; 0.1 M aluminium lactate buffer, pH 3.2, 3 M urea; 1 mm × 18 cm × 28 cm slabs; 4 h at 20 V/cm and 5° C). Gels were stained with 0.05% Nigrosine in methanol/H_2O/acetic acid (5:5:1, by volume).

Results

Characterization of a cDNA clone encoding trypsin inhibitor CMe

A cDNA library derived from barley endosperm at 14 days after pollination (dap) was screened with a 17 nucleotide

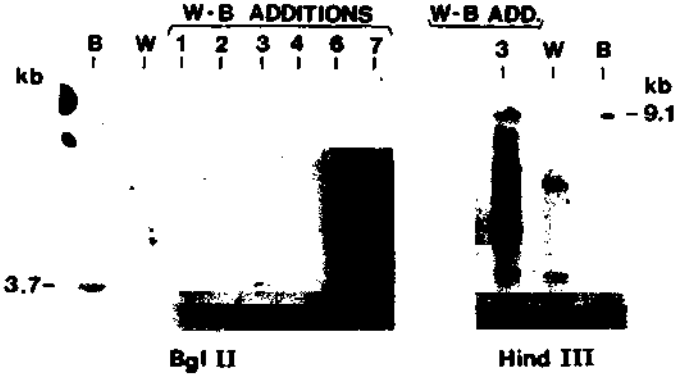

Fig. 1. Southern blot analysis, using the insert in clone λCMe as a probe, of DNA from the following stocks: Betzes barley (B); Chinese Spring (W); Chinese Spring wheat/Betzes barley addition lines (1, 2, 3, 4, 6, 7). Line 5 was not available. DNA digested with restriction endonuclease *Bgl*II was hybridized at 65° C. Confirmation of the chromosome assignment was done with DNA digested with *Hind*III and the hybridization was at lower stringency (58° C). λDNA digested with *Hind*III (Boehringer) was used as molecular weight marker

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                                                                                                                    120
TTGATAACAATGGCGTTCAAGTACCAGCTCCTCCTGTCGGCCGCCGTCATGCTCGCCATTCTCGTCGCCACTGCCACCAGTTTCGGGGATTCGTGTGCTCCAGGGGATGCGTTGCCACAC
        M  A  F  K  Y  Q  L  L  L  S  A  A  V  M  L  A  I  L  V  A  T  A  T  S  F  G  D  S  C  A  P  G  D  A  L  P  H
        └──► Leader                                                                   └──► Mature Protein

                                                                                                                    240
AACCCTCTCAGAGCCTGCCGCACCTACGTGGTCAGTCAAATCTGCCACCAAGGCCCCAGACTCCTCACCTCGGACATGAAGAGGCGGTGTTGCGACGAGCTGTCGGCCATCCCGGCGTAC
N  P  L  R  A  C  R  T  Y  V  V  S  Q  I  C  H  Q  G  P  R  L  L  T  S  D  M  K  R  R  C  C  D  E  L  S  A  I  P  A  Y

                                                                                                                    360
TGTAGGTGCGAAGCGCTGCGTATCATCATGCAAGGGGTAGTAACTTGGCAGGGTGCGTTCGAGGGTGCCTACTTCAAGGACTCGCCCAACTGCCCTAGGGAGAGGCAAACGAGCTACGCC
C  R  C  E  A  L  R  I  I  M  Q  G  V  V  T  W  Q  G  A  F  E  G  A  Y  F  K  D  S  P  N  C  P  R  D  R  Q  T  S  Y  A
                  Probe

                                                                                                                    480
GCCAACCTCGTCACCCCGCAGGAGTGCAACCTAGGGACCATCCACGGCAGCGCGTACTGCCCCGAACTGCAGCCCGCATATTGAGTGGTCTTGTAATAAGTTCTAACGACTACCTCGATC
A  N  L  V  T  P  Q  E  C  N  L  G  T  I  H  G  S  A  Y  C  P  E  L  Q  P  G  Y  *        *  *

ATGAATAAGCATGGTGCATCGATCAATGGACCAATCGATTGTATGCATCTATGCCCATGTGTGATGTGTGTTAAATCGTAATAAAGAGCATCAGAGTATTTTC(A)n
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Fig. 2. Nucleotide sequence and deduced amino acid sequence corresponding to the longest open reading frame of the insert in clone λCMe. The beginning of the leader sequence and of the mature protein are indicated. A *bracket* spans the region hybridizing with the probe. The only position that differs from the known amino acid sequence of trypsin inhibitor CMe (Odani et al. 1983) is *boxed*. The AATAA polyadenylation signal is *underlined*. Stop codons are indicated with *asterisks*

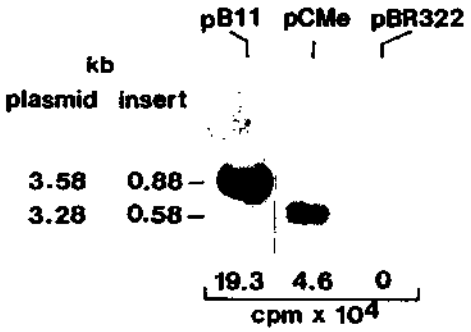

Fig. 3. Amount of CMe mRNA relative to B-hordein mRNA in the endosperm of Bomi barley 18 days after pollination. Linearized plasmids were subjected to electrophoresis, transferred to a nylon membrane (Hybond N, Amersham), and hybridized with ^{32}P-RNA. After autoradiography, the bands were excised and their radioactivity estimated by liquid scintillation spectrometry. To correct for the difference in insert size the radioactivity (4.6×10^4 cpm) corresponding to pCMe was multiplied by the factor 0.88/0.58

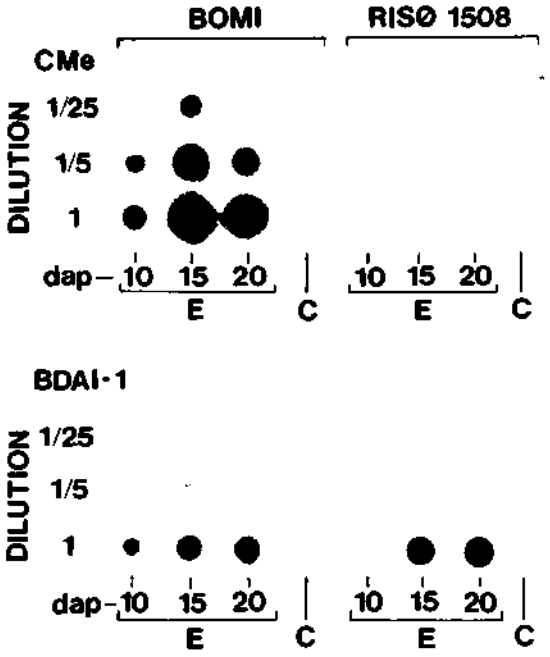

Fig. 4. Dot-blot quantitation of mRNAs corresponding to trypsin inhibitor CMe and barley dimeric α-amylase inhibitor BDAI-1 in developing endosperm from barley cv. Bomi and its mutant Risø 1508. Dilution 1 corresponds to 10 µg of total RNA. After the dot-blot experiment, the probe was washed and the filter hybridized again with a probe for rRNA. The dots were then excised and counted. Differences of total RNA between samples were <10%. Endosperms (E) at 10, 15, and 20 days after pollination (dap) were analysed together with coleoptiles (C) as negative controls

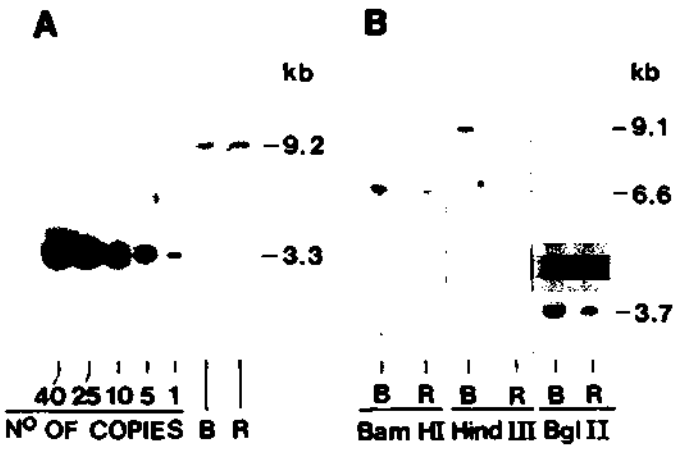

Fig. 5A. Number of copies of gene *CMe* in Bomi barley (B) and its mutant Risø 1508 (R). DNA digested with the restriction endonuclease *Xba*I. B Restriction patterns obtained with the indicated restriction endonucleases. Hybridization was carried out at 65° C

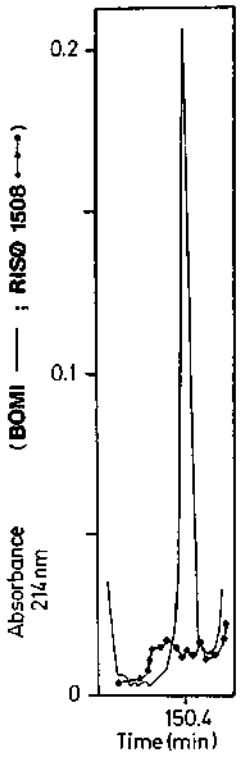

Fig. 6. HPLC analysis of protein CMe in Bomi barley and its mutant Risø 1508. The same amount of dry endosperm (ca. 30 mg) was extracted with NaCl and analysed in both stocks. The CMe peak in Bomi is approximately equivalent to that obtained with 25 µg of pure CMe protein. Only protein CMe was detected by two-dimensional electrophoresis of the HPLC peak

T T
A A A
mixed probe (ATCATCATGCAGGGXGT) corresponding to positions 61–66 of the published amino acid sequence of trypsin inhibitor CMe (Odani et al. 1983). The insert of a clone which hybridized with this probe, λCMe, was used for the Southern analysis of wheat-barley chromosomal addition lines, obtained by Islam et al. (1978), and thus found to hybridize with the DNA of barley chromosome 3H, as shown in Fig. 1. This chromosome had been previously proposed as the site of gene *CMe*, based on the electrophoretic analysis of the protein (Salcedo et al. 1984; Hejgaard et al. 1984). The insert of clone λCMe was subcloned and sequenced (Fig. 2). The deduced amino acid sequence corresponding to the longest open reading frame consisted of a typical signal peptide at the N-terminus, followed by a sequence that was identical to that obtained by direct protein sequencing of trypsin inhibitor CMe (Odani et al. 1983), except for a Leu/Ile substitution at position 59 of the mature protein. The presence of a signal

peptide was expected from previous observations concerning other members of the same protein family (Paz-Ares et al. 1983, 1986; Lazaro et al. 1988a, b).

The subcloned CMe cDNA (pCME) was blotted onto a nitrocellulose filter together with a B-hordein cDNA and hybridized with radioactively labeled total RNA from Bomi endosperm (18 dap) and, after autoradiography, the appropriate zones of the filter were excised and counted (Fig. 3). The amount of CMe mRNA was thus estimated at about one-third of that of the mRNAs which hybridized with the B-hordein cDNA.

Control of gene CMe *by the* lys 3a *locus*

Relative steady-state levels of the *CMe* mRNA were determined during endosperm development, in cv. Bomi and its mutant Risø 1508, by dot-blot hybridization with CMe cDNA as the probe (Fig. 4, top). Maximum levels, which were reached before 20 dap, were about 100-fold lower in the mutant with respect to the wild type. As a control, the same RNA samples were hybridized with a cDNA which encoded a different member of the same protein family, the barley dimeric inhibitor of heterologous α-amylases BDAI-1 (Lazaro et al. 1988b). In that case, the mRNA level was higher in the mutant than in the wild type (Fig. 4, bottom).

The number of copies per haploid genome were 1–2 in both mutant and wild type (Fig. 5A), and the restriction patterns obtained with four restriction endonucleases were also identical in both stocks (Fig. 5A and B).

To resolve the apparent contradiction between the analysis by two-dimensional electrophoresis, which had not de-

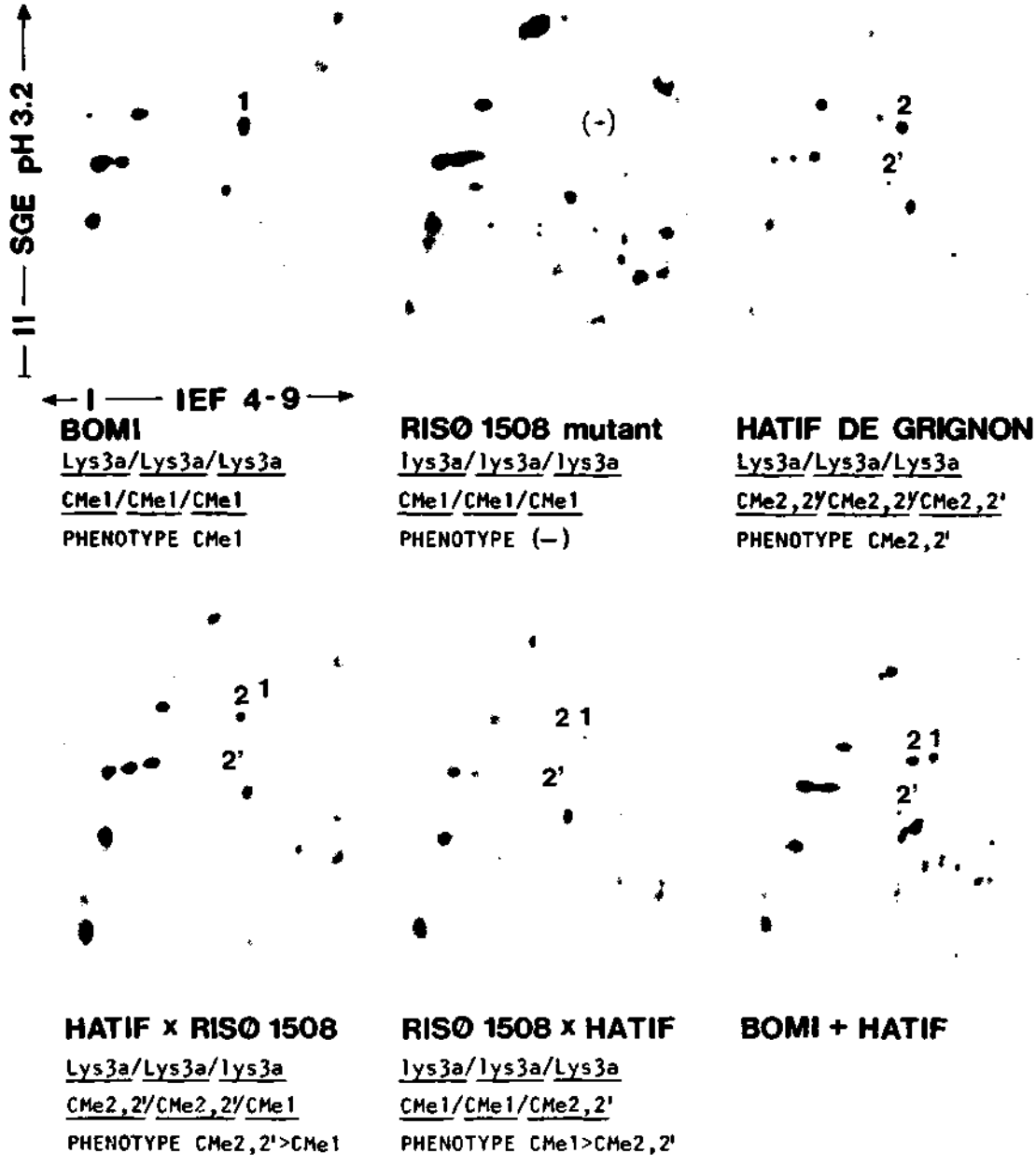

Fig. 7. Analysis by combined isoelectrofocusing (IEF pH 4–9) and starch-gel electrophoresis (SGE pH 3.2) of 0.5 M NaCl extracts of the indicated stocks. Genotypes for the *CMe* and *lys 3a* loci are listed in each case. Spots corresponding to variant CMe1 and CMe2,2′ are indicated in the two-dimensional maps by 1, 2, and 2′, respectively

tected any CMe inhibitor in the mutant (Salcedo et al. 1984), and that carried out by HPLC, which estimated the inhibitor level in the mutant as 20% that of the wild type (Lazaro et al. 1985), the analysis was repeated using a different HPLC column. As shown in Fig. 6, a Vydac TP-RP-C4 column resolved protein CMe from minor overlapping proteins and allowed estimation of the maximum possible amount of inhibitor in the mutant as less than 2%–3% of the wild-type amount.

To ascertain that the mutagenesis treatment used to obtain mutant Risø 1508 had not affected gene *CMe* itself, reciprocal crosses were carried out between the mutant and barley cv. Hatif de Grignon, which had been previously shown by Salcedo et al. (1984) to have a pair of variants of the inhibitor (CMe-2-2′), which did not overlap with the CMe-1 variant prevailing in most *H. vulgare* cultivars surveyed, including cv. Bomi. Seeds from the F1 of the two crosses were analysed by two-dimensional electrophoresis and in both cases the CMe-1 variant coexisted with the CMe-2-2′ ones (Fig. 7).

Discussion

The trypsin inhibitor sequenced by Odani et al. (1983) and protein CMe (Salcedo et al. 1984) had been found to be identical (Lazaro et al. 1985), and the corresponding gene had been located in chromosome 3H (Hejgaard et al. 1984; Salcedo et al. 1984). Besides trypsin inhibitor CMe, a second homologous trypsin inhibitor, designated CMc, had been identified in barley endosperm (Barber et al. 1986) and its gene assigned to chromosome 7H (Salcedo et al. 1984). The two trypsin inhibitors belong to a family which also includes inhibitors of heterologous α-amylases (Garcia-Olmedo et al. 1987). The cloned cDNA reported here (clone λCMe) clearly corresponds to the first inhibitor on the basis of the amino acid sequence deduced from it and the chromosomal location of DNA sequences with which it hybridizes.

In mature kernels of mutant Risø 1508, trypsin inhibitor CMe is either absent or, at most, represents less than 2%–3% of the amount present in the wild type. This is in line with the observed steady-state levels of the corresponding mRNAs during endosperm development, which are about 1% in the mutant with respect to the wild type. The lower expression level is not brought about either by changes in copy number of the structural gene, as was the case for B-hordein in the *hor-2ca* mutation (Kreis et al. 1983), or by a major rearrangement of the gene, as judged by the unaltered restriction patterns in the mutant. Furthermore, the functional and regulatory properties of the gene have not been affected in the mutant because normal expression is observed in endosperms from reciprocal crosses between Risø 1508 and cv. Hatif de Grignon. Endosperm is a triploid tissue, where two genome complements are contributed by the mother. Thus, endosperms from the F1 (Risø 1508 × Hatif) have two doses of the CMe-1 allele and one of the CM2-2′ allele at the *CMe* locus in chromosome 3H, and two doses of the mutant allele and one dose of the wild-type one at the regulatory *lys 3a* locus in chromosome 7 (synonym 5H). One dose of the wild-type allele at the regulatory locus is enough for the accumulation of normal levels of the CMe-1 protein. All this evidence indicates that the expression of gene *CMe* is regulated in *trans* by the gene at the *lys 3a* locus.

The effect of the *lys 3a* mutation on gene *CMe* seems to be rather specific because it does not negatively affect expression of the gene for the homologous amylase inhibitor BDAI-1, nor that of trypsin inhibitor CMc or those of the other known members of the same protein family (Salcedo et al. 1984; Lazaro et al. 1985). Other genes which have been reported to be negatively affected by the *lys 3a* mutation, besides those encoding different hordeins, are those for protein Z and β-amylase (Balasaraswathi et al. 1984; Kreis et al. 1987). In contrast to these two proteins, which are greatly increased by the *lys* mutation in Hiproly barley (Hejgaard and Boisen 1980; Boisen et al. 1981; Hejgaard 1982), trypsin inhibitor CMe is also markedly decreased by the latter mutation (Lazaro et al. 1985). A structural and functional investigation of the promoter of gene *CMe* should contribute to a characterization of the *lys 3a* regulatory locus. Since gene *CMe* is expressed in the first half of endosperm development, while hordeins, protein Z and β-amylase are mainly accumulated in the second half, the present results indicate that the *lys 3a* locus must be active during most of the developmental period.

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